

SITE-SPECIFIC CHEMICAL MODIFICATION OF HIV gp41-DERIVED PEPTIDES

FIELD OF INVENTION

The present invention relates to a method for the site-specific chemical
5 modification of an HIV gp41-derived peptide such that, during peptide synthesis, added is
one or more amino acids having an amine group chemically protected with a chemical
protecting agent, leaving one or more free amine groups of the synthetic peptide chosen
to be unprotected so as to be chemically reactive ("free"). The resultant synthetic peptide
may then be covalently coupled to an amine-reactive polymer in forming a substantially
10 homogeneous conjugate comprised of HIV gp41-derived peptide to which, in selected
and specific site(s) of the synthetic peptide, is covalently coupled the polymer.

BACKGROUND OF THE INVENTION

It is now well known that cells can be infected by HIV through a process by which
15 fusion occurs between the cellular membrane and the viral membrane. The generally
accepted model of this process is that the viral envelope glycoprotein complex
(gp120/gp41) interacts with cell surface receptors on the membranes of the target cells.
Following binding of gp120 to cellular receptors (e.g., CD4 in combination with a
chemokine co-receptor such as CCR-5 or CXCR-4), induced is a conformational change
20 in the gp120/gp41 complex that allows gp41 to insert into the membrane of the target cell
and mediate membrane fusion.

The amino acid sequence of gp41, and its variation among different strains of HIV,
is well known. FIG.1 is a schematic representation of the generally accepted functional
domains of gp41 (note the amino acid sequence numbers may vary slightly depending on
25 the HIV strain). The fusion peptide (fusogenic domain) is believed to be involved in
insertion into and disruption of the target cell membrane. The transmembrane domain,
containing the transmembrane anchor sequence, is located at the C-terminal end of the
protein. Between the fusion peptide and transmembrane anchor are two distinct regions,
known as heptad repeat (HR) regions, each region having a plurality of heptads. The
30 amino acid sequence comprising the HR1 region and the amino acid sequence
comprising the HR2 region are each highly conserved regions in the HIV-1 envelope
protein. The HR1 region, nearer to the N-terminal end of the protein than the HR2 region,
has been generally described as comprising amino acid residues of SEQ ID NO:1, or
polymorphisms thereof (see, e.g., FIG. 2). The HR2 region has been generally described
35 as comprising amino acid residues of SEQ ID NO:2, or polymorphisms thereof (see, e.g.,
FIG. 3). As further shown in FIG.1, the HR regions have a plurality of 7 amino acid
residue stretches or "heptads" (the 7 amino acids in each heptad designated "a" through

"g"), with a predominance of hydrophobic residues at the first ("a") and fourth ("d") positions, charged residues frequently at the fifth ("e") and seventh ("g") positions, and with the amino acids in the "a" position and "d" position being primary determinants that influence the oligomeric state and strand orientation.

5 It was discovered that peptides derived from the native sequence of either the HR1 region ("HR1 peptides") or HR2 region ("HR2 peptides") of HIV gp41 inhibit transmission of HIV to host cells both in *in vitro* assays and in *in vivo* clinical studies. For example, HR2 peptides, as exemplified by DP178 (also known as T20, enfuvirtide, and Fuzeon® ; SEQ ID NO:3), T651 (SEQ ID NO:4), T649 (SEQ ID NO:5), blocked infection
10 of target cells with potencies of 0.5 ng/ml (EC50 against HIV-1_{LAI}), 5 ng/ml (IC50; HIV-1 IIIB), and 2 ng/ml (IC50; HIV-1 IIIB), respectively. Efforts have been made to improve the biological activity of HIV gp41-derived peptides, such as by trying to stabilize the helical structure of the peptide. Various efforts have been also been made to improve the pharmacological properties of HIV gp41-derived peptides.

15 Polymers have been used extensively to improve the pharmacokinetics and pharmacodynamics (and hence, drug performance) of drugs such as peptides, proteins, and small molecules. The most widely used polymer for pharmaceutical applications is polyethylene glycol ("PEG"). "PEGylation" is the process by which the drug is chemically modified to result in the covalent attachment ("coupling") of one or more PEG molecules
20 to the drug (depending on how many sites are available on the drug to interact with, and be conjugated to PEG). The improved pharmacological and biological properties associated with PEGylation of drugs are well known in the pharmaceutical art. For example, PEGylation can increase therapeutic efficacy by means including, but not limited to, reducing degradation by proteolytic enzymes and thereby increasing drug
25 concentration; increasing the size of the drug to which it is attached, thereby improving drug biodistribution; and shielding antigenic epitopes in reducing immunogenicity where desired. By increasing the therapeutic efficacy, reduced may be the frequency of dosing and/or the amount of drug need to achieve a therapeutic effect.

30 PEG, as a linear polyether, has a general structure of:

$\text{HO}-(\text{CH}_2-\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2-\text{OH}$ where n can typically range from about 10 to about 2000.

PEG, as a branched polyether, has a general structure of:

35 PEG-T-PEG

|
Z

wherein T is a linker or molecular bridge linking the PEG molecules, and Z is the

functional group with chemically reactive moiety.

Many of the PEG modifications, in forming PEG derivatives (PEG and PEG derivatives are known in the art as "PEG"), are directed to the end groups ("functionalities") in adding or varying their chemically reactive functionalities to be used to covalently attach the PEG molecule to a drug. Various PEG derivatives are well known in the art. To couple PEG to a drug, typically a functionality of the PEG molecule needs to be activated so as to be chemically reactive. The type and specificity of functionality is based upon the choice of chemically reactive group on the drug to which the PEG molecule is to be coupled. Most commonly for proteins and peptides, the chemically reactive group is present on an amino acid selected from the group consisting of an internal amino acid having a side chain with a free chemically reactive group (e.g., including, but not limited to, lysine, cysteine, glutamic acid, serine, threonine, and the like), the N-terminal amino acid (having a N-terminal amine group, or a side chain amine group, as a free chemically reactive group), a C-terminal amino acid (having a C-terminal carboxylic acid, or side chain amine group, as a free chemically reactive group), and a combination thereof. Of the sites of a peptide to be coupled to PEG, most frequently chosen is the N-terminal amine group ("alpha amine") of the peptide's N-terminal amino acid, and the epsilon amine group ("epsilon amine") of a lysine (a lysine found within the amino acid sequence which is not the N-terminal amino acid or the C-terminal amino acid of the peptide) or an epsilon amine group of lysine when the lysine is present in a peptide as a N-terminal amino acid or as a C-terminal amino acid.

However, a problem arises with this standard strategy for PEGylation. Lysine is one of the most prevalent amino acids in proteins. As related to HIV gp41, there are multiple lysine residues in the amino acid sequences of the HR1 region and the HR2 region (see, e.g., FIGs. 1-3). With respect to HIV gp41-derived peptide T20 (SEQ ID NO:1), for example, there are two internal lysine residues in this 36 amino acid residue peptide. Thus, with a plurality of lysine residues in the amino acid sequence (hence, a plurality of side chain amines (epsilon amines) available to be reactive with activated PEG containing amine-reactive functionality) and an alpha amine, there exists several sites to which the activated PEG with an amine reactive functionality, can be covalently coupled. The result of standard PEGylation of such a peptide is a heterogeneous mixture comprised of a population of several conjugates varying in the number of PEG molecules attached and in the sites of attachment. Heterogeneity of such a synthetic peptide-polymer conjugate is often an undesirable result. This is because the pharmacological and/or biological properties associated with PEGylation of peptides can be dependent on factors such as (a) the number of PEG molecules attached to the peptide, and (b) the location of the sites on the peptide to which PEG is coupled. For example, *in vitro*

biological activity of PEGylated human growth hormone-releasing factor depended on both the site and degree of PEGylation. Further, from standard PEGylation, it is very difficult, if possible at all, to separate out the species of peptide-polymer conjugate (with the desired number of PEG molecules and desired site(s) of attachment) from a
5 heterogeneous mixture using conventional separation techniques known in the art. Such separation attempts add to the expense, time, and reagents needed for producing the peptide-polymer conjugate of the desired species. Multiple lysine residues in the amino acid sequence of a peptide to be PEGylated are perceived as such a problem that one method of site-specific PEGylation was developed which involved replacing the lysine
10 residues with amino acids other than lysine, and which lack a side chain having a free amine.

Thus, in the formation of conjugates comprised of an HIV gp41-derived peptide (containing one or more internal amino acid residues having a side chain amine in its amino acid sequence) and polymer, there is a need for a site-specific modification of the
15 synthetic peptide so that produced is a synthetic peptide containing one or more selected amino acids having a side chain amine chemically protected with a chemical protecting agent, and one or more amino acids having an unprotected, free amine available. Accordingly, a polymer may be covalently coupled only to a specific site, or specific sites, on the synthetic peptide, as selected by a person performing the synthesis and
20 conjugation. Additionally, when the polymer to be conjugated is branched, there is a need for a site-specific modification of the synthetic peptide so as select only one free amine to be covalently coupled to polymer, in avoiding multiple branches of the same molecule of polymer conjugating to (and cross-linking of) the same molecule of synthetic peptide. More specifically, in an HIV gp41-derived peptide containing more than one
25 chemically reactive ("free") amine group which is available for coupling to a polymer having amine-reactive functionality(s), it is desirable to chemically protect one or more selected amine groups, leaving the unprotected, free amine group(s) available for covalently coupling to polymer. Additionally, it would be advantageous to provide an HIV gp41-derived peptide which has PEG coupled in a site-specific manner to one or more
30 selected sites (i.e., in one or more selected amino acid positions) in the synthetic peptide.

SUMMARY OF THE INVENTION

The present invention relates to a method for site-specific chemical modification of an HIV gp41-derived peptide during synthesis of the peptide, wherein the synthesized
35 peptide has one or more amino acids having a side chain amine. The method comprises incorporating into the peptide, or a fragment thereof, during synthesis, at least one amino acid selected to have its side chain amine chemically reacted with a chemical protecting

agent which protects the side chain amine from subsequent chemically reactivity with an amine-reactive functionality; and at least one amino acid having an amine unprotected and free for reacting with an amine-reactive functionality, wherein the free amine is selected from the group consisting of an N-terminal amine, a side chain amine, and a combination thereof. Also, produced from this method is an isolated HIV gp41-derived peptide having one or more amino acids containing a side chain amine, wherein at least one amino acid has its side chain amine chemically reacted with a chemical protecting agent which protects the side chain amine from subsequent chemically reactivity with an amine-reactive functionality; and at least one amino acid of the synthetic peptide has an amine unprotected and free for reacting with an amine-reactive functionality, wherein the free amine is selected from the group consisting of an N-terminal amine, a side chain amine, and a combination thereof.

The present invention relates to a method for producing a substantially homogeneous conjugate comprised of HIV gp41-derived peptide and polymer, wherein the HIV gp41-derived peptide has, incorporated into its amino acid sequence during synthesis, one or more amino acids having a side chain amine which has been selected to be blocked by a chemical protecting agent, allowing only the desired (selected) unblocked free amine group(s) of the synthetic peptide to be available for reaction with a polymer containing a amine-reactive functionality, in covalently coupling the synthetic peptide to the polymer at only specific site(s) (amino acid position(s) of the synthetic peptide) containing a free amine. The present invention also relates to a substantially homogeneous conjugate comprised of HIV gp41-derived peptide and polymer produced according to this method according to the present invention.

The present invention also provides a method for site-specific PEGylation of an HIV gp41-derived peptide, wherein PEG is covalently coupled in a site-specific manner to an HIV gp41-derived peptide. More particularly, the HIV gp41-derived peptide, having had incorporated into its amino acid sequence at selected amino acid positions during synthesis, one or more amine groups (e.g., one or more of: an alpha amine or an epsilon amine(s)) which is blocked with a chemical protecting agent from chemically reactive with amine-reactive functionality of PEG during PEGylation, thereby leaving available for PEGylation only the free amine group(s) in selected amino acid positions (through chemical modification) of the synthetic peptide to be covalently coupled to PEG. Using the method of the present invention, provided is a substantially homogeneous composition comprising a PEGylated HIV gp41-derived peptide containing one or more (as selected in performing the method of site-specific chemical modification) amine groups conjugated to PEG.

The methods of the present invention may further comprise removal of the

chemical protecting agent (in a "deprotection" step) in providing a substantially homogeneous conjugate comprised of an HIV gp41-derived peptide which is conjugated to a polymer at only specific site(s) of the synthetic peptide, as selected in performance of the method of the present invention (e.g., through use of chemical protection), wherein
5 such conjugate retains substantial anti-HIV activity (as compared to the anti-HIV activity of the synthetic peptide when not conjugated to polymer). The present invention also provides for a method of treating HIV infection (preferably, HIV-1 infection) comprising administering to an HIV-infected individual a pharmaceutical composition comprising a substantially homogeneous conjugate comprised of an HIV gp41-derived synthetic
10 peptide site-specifically coupled (e.g., conjugated) to polymer. Preferably, the pharmaceutical composition is in an amount effective to inhibit transmission of HIV to a target cell, and/or in an amount effective to inhibit gp41-mediated fusion of HIV to a target cell. Also provided is a method for inhibition of transmission of HIV to a cell, comprising contacting the virus in the presence of a cell with the substantially homogeneous
15 conjugate of polymer and synthetic peptide according to the present invention in an amount effective to inhibit infection of the cell by HIV. Additionally, provided is a method for inhibition of transmission of HIV to a cell, comprising adding to the virus and the cell an amount of the substantially homogeneous conjugate of polymer and synthetic peptide according to the present invention effective to inhibit infection of the cell by HIV. Also
20 provided is a method for inhibiting HIV fusion (e.g., a process by which HIV gp41 mediates fusion between the viral membrane and cell membrane during infection by HIV of a target cell), comprising contacting the virus in the presence of a cell with an amount of the substantially homogeneous conjugate of polymer and synthetic peptide according to the present invention effective to inhibit HIV fusion. These methods may be used to
25 treat HIV-infected individuals.

The present invention also provides the use of a substantially homogeneous conjugate of polymer and synthetic peptide, produced by the method according to the present invention, in the manufacture of a medicament for use with in therapy of HIV infection (e.g., used in a method of inhibiting transmission of HIV, a method of inhibiting
30 HIV fusion, or a method of treating HIV infection), as described herein. The medicament is preferably in the form of a pharmaceutical composition comprising a substantially homogeneous conjugate of polymer and synthetic peptide according to the present invention together with a pharmaceutically acceptable carrier.

The above descriptions, features, and advantages of the present invention will be
35 apparent in the following Detailed Description of the Invention when read in conjunction with accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic of HIV-1 gp41 showing the heptad repeat 1 region (HR1) and heptad repeat 2 region (HR2) along with other functional regions of gp41. Exemplary amino acid sequences corresponding to HR1 and HR2, and the amino acid position numbering, are shown for purposes of illustration and in relation to gp160, strain HIV_{III}B.

FIG. 2 shows a comparison of the sequences contained within the HR1 region of HIV-1 gp41 for purposes of illustration, and not limitation, as determined from various laboratory strains and clinical isolates, wherein illustrated are some of the variations in amino acid sequence (e.g., polymorphisms), as indicated by the single letter amino acid code.

FIG. 3 shows a comparison of the sequences contained within the HR2 region of HIV-1 gp41 for purposes of illustration, and not limitation, as determined from various laboratory strains and clinical isolates, wherein illustrated are some of the variations in amino acid sequence (e.g., polymorphisms), as indicated by the single letter amino acid code.

FIG. 4 is a schematic showing synthesis of an HIV gp41-derived peptide using a fragment condensation approach, wherein: the numbers represent respective amino acid positions relative to the synthesized HIV gp41-derived peptide; "K" represents a lysine internal to the sequence of the synthetic peptide, or to a fragment used in the synthesis of the synthetic peptide; "Ac" represents acetylation of the N-terminus; and "NH" represents amidation of the C-terminus.

FIG. 5 is a schematic showing synthesis of modified HIV gp41-derived peptide, and a conjugate of polymer and synthetic peptide, according to the present invention, wherein: the numbers represent respective amino acid positions relative to the synthesized HIV gp41-derived peptide; "K" represents a lysine internal to the sequence of the synthetic peptide, or to a fragment used in the synthesis of the synthetic peptide; "Ac" represents acetylation of the N-terminus; "NH" represents amidation of the C-terminus; "X" represents a chemical protecting agent which is coupled to an epsilon amine of a selected amino acid in selectively blocking the amino acid side chain from further chemical reactivity; and "I" represents a polymer which has specificity for chemically coupling with a free amine group, and becomes conjugated to a free amine of an amino acid which is not coupled to a chemical protecting agent in a site-specific chemical modification. Produced are: an isolated HIV gp41-derived peptide having at least one amine group (e.g., epsilon amine of the lysine residue at amino acid position 18) chemically protected by a chemical protecting agent; and a substantially homogeneous conjugate comprised of HIV gp41-derived peptide and polymer, with polymer conjugated site-specifically to the synthetic peptide (e.g., at the epsilon amine of the lysine residue at

amino acid position 28).

FIG. 6 is a schematic showing synthesis of modified HIV gp41-derived peptide, and a conjugate of polymer and synthetic peptide, according to the present invention, wherein: the numbers represent respective amino acid positions relative to the synthesized HIV gp41-derived peptide; "K" represents a lysine internal to the sequence of the synthetic peptide, or to the fragment used in the synthesis of the synthetic peptide; "Ac" represents acetylation of the N-terminus; "NH" represents amidation of the C-terminus; "X" represents a chemical protecting agent which is coupled to an epsilon amine in selectively blocking the amino acid side chain from further chemical reactivity; and "I" represents a polymer which is conjugated to an amine of an amino acid which is not coupled to a chemical protecting agent in a site-specific chemical modification. Produced are: an isolated HIV gp41-derived peptide having at least one amine group (e.g., epsilon amine of the lysine residue at amino acid position 28) chemically protected by a chemical protecting agent; and a substantially homogeneous conjugate comprised of HIV gp41-derived peptide and polymer, with polymer conjugated site-specifically to the synthetic peptide (e.g., at the epsilon amine of the lysine residue at amino acid position 18).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "individual", when used herein for purposes of the specification and claims, means a mammal, and preferably a human.

The term "target cell", when used herein for purposes of the specification and claims, means a cell capable of being infected by HIV. Preferably, the cell is a human cell or are human cells; and more preferably, human cells capable of being infected by HIV via a process including membrane fusion.

The term "pharmaceutically acceptable carrier", when used herein for purposes of the specification and claims, means a carrier medium that does not significantly alter the biological activity of the active ingredient (e.g., a conjugate of polymer and synthetic peptide according to the present invention) to which it is added. A pharmaceutically acceptable carrier includes, but is not limited to, one or more of water, buffered water, saline, 0.3% glycine, aqueous alcohols, isotonic aqueous solution; and may further include one or more substances such as glycerol, oils, salts such as sodium, potassium, magnesium and ammonium, phosphonates, carbonate esters, fatty acids, saccharides (e.g., mannitol), polysaccharides, excipients, and preservatives and/or stabilizers (to increase shelf-life or as necessary and suitable for manufacture and distribution of the composition). Preferably, the pharmaceutically acceptable carrier is suitable for

intravenous, intramuscular, subcutaneous or parenteral administration.

By the term "amino acid" is meant, for purposes of the specification and claims and in reference to the synthetic peptides used in the present invention, to refer to a molecule that has at least one free amine group and at least one free carboxyl group. The amino acid may have more than one free amine group, or more than one free carboxyl group, or may further comprise one or more free chemical reactive groups other than an amine or a carboxyl group (e.g., a hydroxyl, a sulfhydryl, etc.). The amino acid may be a naturally occurring amino acid (e.g., L-amino acid), a non-naturally occurring amino acid (e.g., D-amino acid), a synthetic amino acid, a modified amino acid, an amino acid derivative, an amino acid precursor, and a conservative substitution. One skilled in the art would know that the choice of amino acids incorporated into a peptide will depend, in part, on the specific physical, chemical or biological characteristics required of the antiviral peptide. Such characteristics are determined, in part, by determination of structure and function (e.g., antiviral activity; as described herein in more detail). For example, the skilled artisan would know from the descriptions herein that amino acids in a synthetic peptide may be comprised of one or more of naturally occurring (L)-amino acid and non-naturally occurring (D)-amino acid. A preferred amino acid may be used to the exclusion of amino acids other than the preferred amino acid.

A "conservative substitution", in relation to amino acid sequence of a synthetic peptide used in the present invention, is a term used hereinafter for the purposes of the specification and claims to mean one or more amino acids substitution in the sequence of the synthetic peptide such that its biological activity is substantially unchanged (e.g., if the peptide inhibits HIV gp41-mediated fusion at a concentration in the nanomolar range before the substitution, after the substitution inhibition of HIV gp41-mediated fusion is still observed in the nanomolar range). As known in the art "conservative substitution" is defined by aforementioned function, and includes substitutions of amino acids having substantially the same charge, size, hydrophilicity, and/or aromaticity as the amino acid replaced. Such substitutions are known to those of ordinary skill in the art to include, but are not limited to, glycine-alanine-valine; isoleucine-leucine; tryptophan-tyrosine; aspartic acid-glutamic acid; arginine-lysine; asparagine-glutamine; and serine-threonine. With particular relevance to the present invention, a conserved substitution is known in the art to also include substituting lysine with ornithine, in providing a free amine group (e.g., epsilon amine). For HIV gp41-derived peptides, such substitutions may also comprise polymorphisms at the various amino acid positions along the relevant HR region (HR1 or HR2) of gp41 found in any one or more of various clades, laboratory strains, or clinical isolates of HIV, which are readily available from public databases and are well known in the art (see also, FIGs. 2 & 3, as illustrative examples).

The term "polymer" when used herein for purposes of the specification and claims, means a polymeric molecule which: (a) is employed in pharmaceutical applications to improve the pharmacological and/or biological properties when conjugated to a drug (and therefore is substantially nontoxic and substantially water soluble); (b) has one or more functionalities which by itself, and/or after activation to become chemically reactive, can be used to covalently couple to a free amine of the drug (e.g., a synthetic peptide) in forming a drug-polymer conjugate. Regarding the latter, the polymer preferably has an amine-reactive functionality for covalently coupling to synthetic peptide. A polymer may include, but is not limited to, polylysines or poly(D-L-alanine)-poly(L-lysine)s, or polyols.

A preferred polyol comprises a water-soluble poly(alkylene oxide) polymer, and can have a linear or branched chain. The term "polyol" is preferably a water-soluble, polyalcohol which may include, but is not limited to, polyethylene glycol ("PEG"), polypropylene glycol ("PPG"), diethylene glycol, triethylene glycol, ethylene glycol, dipropylene glycol, copolymers comprising PPG (e.g., ethylene glycol/PPG), copolymers comprising PEG (e.g., PEG/PPG), mPEG (monomethoxy-poly(ethylene) glycol), and the like. A polyol encompasses both homopolymers and copolymers, and further may have a structure comprising a branched structure or linear structure as known to those skilled in the art. Preferably, the polymer is substantially non-toxic when used for *in vivo* applications in individuals. In a preferred embodiment, the polymer has a molecular weight in the range between about 200 daltons to about 40,000 daltons; and in a more preferred embodiment, the polymer has a molecular weight range between about 400 daltons to about 10,000 daltons. A preferred polymer for application in the present invention comprises a polyethylene glycol ("PEG"), and a more preferred polymer for application in the present invention comprises a polyethylene glycol having a molecular weight range, wherein the molecular weight range is no less than about 400 daltons and is no more than about 20,000 daltons. As described previously herein, there are various forms of PEG that typically differ in the end groups or chemically reactive functional groups to be used to covalently attach the PEG molecule to a drug. Various PEGs are well known in the art. A preferred PEG, for use in coupling to one or more unprotected amine groups of the synthetic peptide in accordance with the present invention, has a chemically reactive group (e.g., "functionality") which can be used covalently couple PEG to the to one or more unprotected amine groups. PEG may include but is not limited to, PEG-tresylate, heterobifunctional PEG, PEG dichlorotriazine, PEG succinimidyl carbonate, PEG benzotriazole carbonate, PEG *p*-nitrophenyl carbonate, PEG trichlorophenyl carbonate, PEG carbonylimidazole, PEG succinimidyl succinate, mPEG succinimidyl propionate, mPEG succinimidyl butanoate, PEG butyraldehyde, mPEG-propionaldehyde, PEG aldehyde, PEG-acetaldehyde, PEG acetaldehyde diethyl acetal, PEG carboxylic acid,

mPEG phenyl ether succinimidyl carbonates, mPEG benzamide succinimidyl carbonates, PEG thioester, linear PEG, branched PEG, and linear forked PEG. A preferred polymer may be applied to the present invention to the exclusion of a polymer other than the preferred polymer.

5 The terms "synthetic peptide" and "HIV gp41-derived peptide" are used synonymously herein, in relation to a peptide employed in the present invention, and for the purposes of the specification and claims, to mean a peptide (a) comprising an amino acid sequence of no less than about 15 amino acids and no more than about 60 amino acid residues in length, and comprises at least a portion of the amino acid sequence
10 (preferably, at least 9 contiguous amino acids) contained in either the HR1 region or HR2 region of gp41 of HIV (more preferably of HIV-1); and (b) capable of inhibiting transmission of HIV to a target cell (preferably, by complexing to an HR region of HIV-1 gp41 and inhibiting fusion between HIV-1 and a target cell), as can be determined by assessing antiviral activity *in vitro* and/or *in vivo*, as will be described in more detail
15 herein. More preferably, the synthetic peptide employed in the present invention may comprise a sequence of no less than 28 amino acids and no more than about 51 amino acids in length, and even more preferably no less than about 36 amino acids and no more than about 51 amino acids in length. The term "isolated" when used in reference to a synthetic peptide means that it is substantially free of components which have not
20 become part of the integral structure of the peptide itself; e.g., such as substantially free of chemical precursors or other chemicals when chemically synthesized, produced, or modified using biological, biochemical, or chemical processes. The synthetic peptide may comprise, in its amino acid sequence, one or more conservative substitutions and/or one or polymorphisms found in the sequence of the relevant region of the HIV gp41, or
25 may comprise one or more amino acid substitutions which are added to stabilize helix structure and/or affect oligomerization; provided that it retains substantial antiviral activity against HIV-1 (e.g., an IC₅₀ in the picomolar to micromolar range). The following are illustrative examples of HIV gp41-derived peptides that can be site-specifically conjugated to polymer in accordance with the present invention. However, a preferred synthetic
30 peptide may be used in the present invention to the exclusion of a synthetic peptide other than the preferred synthetic peptide. As apparent to one skilled in the art and from the teachings herein, a lysine in the amino acid sequence of a synthetic peptide may be substituted with another amino acid (naturally occurring or not naturally occurring) having a side chain with a free amino group (e.g., epsilon amine). Ornithine is an illustrative
35 example of such amino acid that may be used to substitute a lysine.

Preferably for use according to the present invention, for a synthetic peptide comprising sequence derived from the HR1 region of HIV-1 gp41, the synthetic peptide

comprises a contiguous sequence of at least 15 amino acid residues in the amino acid sequence of SEQ ID NO:1, or polymorphisms thereof, as key determinants in this portion of the HR1 region (e.g., such as, noted by single letter amino acid designation, NNLLRAIEAQQHLLQLTVWG IKQLQARI LAVERYLKD which is amino acid residue 18 to amino acid residue 54 of SEQ ID NO:1) have been found to influence structure, and biochemical and antiviral parameters described herein. Note that there are two lysine residues internal to this portion of the HR1 region, one or more of which may be used for site-specifically coupling to a polymer according to the present invention. A preferred example of a synthetic peptide derived from the HR-1 region of HIV gp41, and as containing the amino acids found in the native sequence of this region, is illustrated as having an amino acid sequence of SEQ ID NO:6. Other examples of a synthetic peptide derived from the HR-1 region of HIV gp41, and as containing the amino acids found in the native sequence of this region, are illustrated as having amino acid sequences of SEQ ID NOs:7-22, and may further comprise an amino acid sequence having at least 95% identity, and more preferably having at least 90% identity, with any one or more of SEQ ID NOs:6-22. More preferably for use according to the present invention, a synthetic peptide derived from the HR1 region of HIV gp41 contains one or more amino acid substitutions (e.g., as compared to the amino acid sequence of SEQ ID NO:1) which preferably enables the synthetic peptide to self-assemble into trimers (e.g., a trimer being comprised of three molecules of synthetic peptide), as disclosed in more detail in co-pending application published as U.S. 20040076637. Examples of a synthetic peptide derived from the HR-1 region of HIV gp41 and which further comprises one or more amino acid substitutions which enable the synthetic peptide to self-assemble into trimers are illustrated as having amino acid sequences of SEQ ID NOs:23-36, and may further comprise an amino acid sequence having at least 95% identity, and more preferably having at least 90% identity, with any one or more of SEQ ID NOs: 23-36. Note that such synthetic peptides have one or more lysine residues internal to this portion of the HR1 region, one or more of which may be chosen to be left unprotected (i.e., its side chain reactive group is not chosen to be coupled to the chemical protecting agent), or may be chosen to be chemically protected, in the site-specific chemical modification according to the present invention.

Preferably for use according to the present invention, for a synthetic peptide comprising sequence derived from the HR2 region of HIV-1 gp41, the synthetic peptide comprises a contiguous sequence of at least amino acid residues 43 to 51 of SEQ ID NO:2 (e.g., QQEKNEQEL), or polymorphisms thereof, as key determinants in this portion of the HR2 region have been found to influence biochemical and antiviral parameters described herein. Note there is one internal lysine residue in this sequence. Illustrative

synthetic peptides derived from the HR2 region include, but are not limited to peptides having the amino acid sequences shown in SEQ ID NOs: 3, 4, 5, 37 to 63, and 175, and may further comprise an amino acid sequence having at least 95% identity, and more preferably having at least 90% identity, with any one or more of SEQ ID NOs: 3, 4, 5, 37 to 63, and 175. Note that such synthetic peptides have one or more internal lysine residues (and/or in the case of SEQ ID NOs. 34, 39, 48, and 175 at the carboxy terminus), one or more of which may be chosen to be left unprotected, or may be chosen to be chemically protected, in the site-specific chemical modification according to the present invention. More preferably for use according to the present invention, a synthetic peptide derived from the HR2 region of HIV gp41 contains one or more amino acid substitutions (e.g., as compared to a relative portion of the amino acid sequence of SEQ ID NO:2) which preferably promotes the helicity and/or helix stability of the synthetic peptide ("helix stabilized peptide") in imparting improved biological activity, as disclosed in more detail in co-pending application PCT/US04/42918. Examples of such helix stabilized synthetic peptides are illustrated as having amino acid sequences of SEQ ID NOs: 64-92, and 113-174, and may further comprise an amino acid sequence having at least 95% identity, and more preferably having at least 90% identity, with any one or more of SEQ ID NOs: 64-92 and 113-174. Other examples of peptides designed for improved helicity and derived from the HR2 region of HIV gp41 may include SEQ ID NOs: 93-95. Note that such helix stabilized peptides have one or more internal lysine residues (and in some cases upwards to 25% of the amino acid sequence of the synthetic peptide), one or more of which may be chosen to be left unprotected, or chosen to be chemically protected, in the site-specific chemical modification according to the present invention.

In another preferred embodiment according to the present invention, the synthetic peptide may comprise a "hybrid" peptide comprising amino acid sequences derived from one or more of HIV-1, HIV-2, and SIV fusion proteins (see, e.g., U.S. Patent No. 6,258,782). Examples of a hybrid synthetic peptide are illustrated as having amino acid sequences of SEQ ID NOs: 96 to 112, and may further comprise an amino acid sequence having at least 95% identity, and more preferably having at least 90% identity, with any one or more of SEQ ID NOs: 96 to 112. Note that such illustrated examples of hybrid synthetic peptides have at least two internal lysine residues, one or more of which may be chosen to be left unprotected, or chosen to be chemically protected, in the site-specific chemical modification according to the present invention.

The term "percent identity", when used herein for purposes of the specification and claims in reference to a sequence used in accordance with the present invention, means that the sequence is compared ("Compared Sequence") to a described or

reference sequence ("Reference Sequence"); wherein a percent identity is determined according to the following formula:

$$\text{percent identity} = [1 - (xC/yR)] \times 100$$

wherein xC is the number of differences between the Reference Sequence and the

5 Compared Sequence over the length of alignment between the Compared Sequence and Reference Sequence wherein (a) each base or amino acid in the Reference Sequence that does not have a corresponding aligned base or amino acid compared to the Compared Sequence, and (b) each gap in the Reference Sequence, and (c) each aligned
10 base or amino acid in the Compared Sequence that is different from an aligned base or amino acid in the Reference Sequence, constitutes a difference; and yR is the number of bases or amino acids in the Reference Sequence over the length of the Compared Sequence with any gap created in the Reference Sequence as a result of alignment also being counted as a base or amino acid. Methods and software for alignment between two predetermined sequences are well known in the art. Thus, for example, a Reference
15 Sequence may be a synthetic peptide according to any one of SEQ ID NOs: 1-175, and a Compared Sequence is an HIV gp41-derived peptide which is compared to the Reference Sequence, in determining an amino acid sequence having at least 95% identity with any one or more of the amino acid sequences of SEQ ID NOs: 1-175.

The term "chemical protecting agent", when used herein for purposes of the
20 specification and claims, means a chemical moiety that: (a) is chemically reactive with a free amine of an amino acid, thereby blocking ("chemically protecting") the amine from reacting with a polymer having a functionality that is amine-reactive; (b) can withstand (e.g., remains chemically reacted with the amine which it is chemically protecting) a
25 deprotection step known to those skilled in the art for removing tBU (t-butyl), Fmoc (9-fluorenylmethoxycarbonyl), Boc (tert-butyloxycarbonyl), or trt (triphenylmethyl(trityl)) from an amino acid; and (c) can be subsequently removed from the amine of the amino acid to which it is chemically reacted, so that the amine becomes unprotected and free for chemical reactivity with an amine-reactive functionality. More particularly, the chemical
30 protecting agent can withstand removal of Fmoc or Boc from a peptide by reagents typically used in the art for such deprotection, including, for example, one or more of: 20% piperidine, 2% DBU (1,8-diazabicyclo[5,4,0]undec-7-ene), 50% to 90% trifluoroacetic acid, a quaternary amine (such as tetrabutyl ammonium fluoride), or an inorganic base such as potassium carbonate. For example, as is discussed in more detail herein, the chemical
35 protecting agent can remain stable (reacted with the amine group) until it is desired to remove the chemical protecting agent, and then the chemical protecting agent is removed in a subsequent, and separate deprotection step (e.g., using 2% hydrazine or other suitable reagent) in yielding a free amine. Such chemical protecting agents are known in

the art to include, but are not limited to, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl ("Dde"), 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde), allyloxycarbonyl ("Alloc"), benzyloxycarbonyl ("Cbz"), and 2-chlorobenzyloxycarbonyl ("2-Cl-Z"). Preferably, in a synthetic peptide, the free amine group reacting with the chemical
5 protecting agent is an N-terminal amine of an N-terminal amino acid, or an amine group of a side chain (e.g., epsilon amine) of an amino acid (whether such amino acid is the N-terminal amino acid, a C-terminal amino acid, or an internal amino acid), or a combination thereof, as determined by the site-specific chemical modification. In a preferred embodiment, the chemical protecting agent is stable to amine bases to which Fmoc, Boc,
10 tBu, trt, or the like are labile.

The term "substantially homogeneous", when used herein for purposes of the specification and claims and in reference to a conjugate comprised of an HIV gp41-derived peptide coupled to a polymer produced according to the present invention, means that at least 90%, and more preferably at least 95%, of the resultant conjugate
15 produced contains the synthetic peptide site-specifically coupled to the polymer as intended (i.e., as a single species) by an orthogonal protection strategy employed (as described in more detail in Example 5 herein), according to the method of the present invention. The conjugate may be further purified using separation technology including, but not limited to, chromatographic techniques known in the art.

20 -----

The present invention provides a method for site-specific chemical modification of an HIV gp41-derived peptide, and provides an isolated HIV gp41-derived peptide containing at least one side chain amine chemically protected, and containing at least one amine (e.g., N-terminal amino acid alpha amine, one or more side chain amines), or a
25 combination thereof) unprotected and free for reactivity with an amine-reactive functionality. The isolated HIV gp41-derived peptide may then be conjugated (covalently coupled) to a polymer at a site-specific location (i.e., at a particular amino acid position, in the synthetic peptide, having a free amine) which is selected (by intentionally not protecting it with a chemical protecting agent) to be coupled to the polymer. Thus, the
30 coupling of the polymer to the synthetic peptide is via one or more free amine groups of the synthetic peptide available for chemical reaction with a polymer having a functionality which is amine-reactive. Accordingly, for example, one molecule of polymer is covalently coupled to an amino acid which is selected to have a free amine in producing a substantially homogeneous conjugate comprised of polymer and HIV gp41-derived
35 peptide. For purposes of illustration, and not limitation, the following schematics demonstrate the methods according to the present invention, an isolated HIV gp41-derived peptide produced by a method according to the present invention, and a

substantially homogeneous conjugate comprised of polymer-synthetic peptide according to the present invention, by using an exemplary HIV gp41-derived peptide known as T20 (SEQ ID NO:3).

5 Schematic 1: The synthetic peptide, being unmodified at the N-terminus after synthesis, has 3 free amine ("NH₂") groups available for coupling to a polymer having an amine reactive functional group: the N-terminal amino acid alpha amine, and two internal lysine residues (labeled K₁ and K₂ for ease of description), each with a side chain having an epsilon amine.

10

Synthetic peptide: $^{2\text{H}}$ -YTSLIHSLIEESQNQQE K₁NEQELLELD K₂WASLWNWF

15 Schematic 2: The synthetic peptide shown in schematic 1 is conjugated to a polymer ("P") having an amine reactive functionality for coupling to the synthetic peptide. A heterogenous population of conjugates is possible from the conjugation process, as follows.

20 Conjugates: **Ī**-YTSLIHSLIEESQNQQE K₁NEQELLELD K₂WASLWNWF

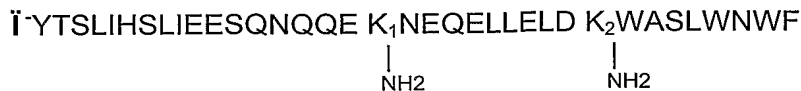
25 ²HN-YTSLIHSLIEESQNQQE K₁NEQELLELD K₂WASLWNWF

[illegible]

I-YTSLIHSLIEESQNQQE K₁NEQELLELD K₂WASLWNWF
| |
NH₂ I

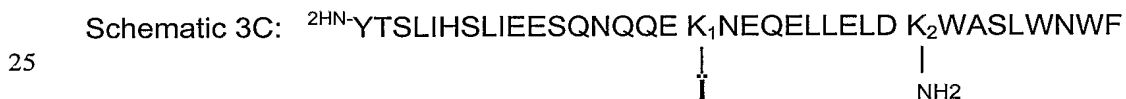
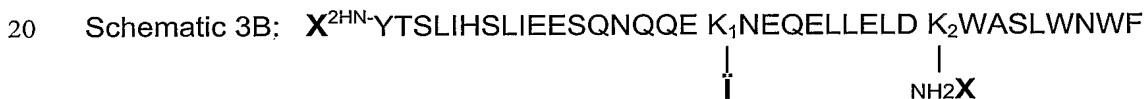
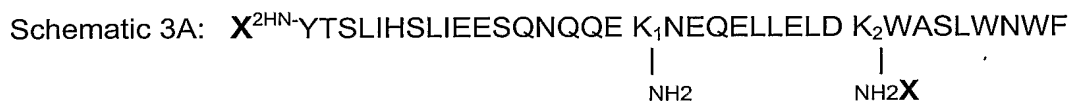
$$^{2\text{HN}}\text{-Y T S L I H S L I E E S Q N Q Q E K}_1\text{ N E Q E L L E L D K}_2\text{ W A S L W N W F}$$

40 ^{2HN}-YTSLIHSLIEESQNQQE K₁NEQELLELD K₂WASLWNWF
| |
NH₂ I



5

Schematic 3: For illustration purposes, only the free amine of internal lysine residue “K₁” is selected to be free for chemical reactivity with an amine reactive functionality. First, the synthetic peptide is synthesized to incorporate amino acids, having amine groups desired to be blocked from reactivity with the polymer, protected by a chemical protecting agent (“X”) in forming an isolated HIV gp41-derived peptide having at least one amino acid with its side chain amine chemically protected (schematic 3A); polymer is then conjugated to such HIV gp41-derived peptide (schematic 3B) at the only amino acid having a free amine (lysine residue K₁); and the chemical protecting agent is subsequently removed from amino acids to which it was chemically reacted, in yielding a substantially homogeneous conjugate (schematic 3C).



The following Examples illustrate the present invention, and should not be construed as limiting the present invention.

EXAMPLE 1

Synthetic peptides may typically be, and have been, synthesized by linear synthesis on a peptide synthesizer using standard solid-phase synthesis techniques and using standard Fmoc peptide chemistry or other standard peptide chemistry. Thus, as shown in synthesis of a fragment illustrated herein, solid phase synthesis or other standard peptide chemistry may be used to synthesize the synthetic peptide, wherein a chemically protected amino acid (e.g., an amino acid having its side chain amine chemically protected as described herein) may be added in the desired amino acid position at the point in synthesis where such amino acid is incorporated into the chain of amino acids to produce the synthetic peptide (as illustrated in Example 6 herein). However, in a preferred embodiment, the HIV gp41-derived peptide to undergo site-

specific chemical modification is synthesized using a fragment condensation approach (see, e.g., FIGs. 4-6), as described in more detail in Example 5 herein. Briefly, 2 or more fragments, each fragment containing amino acid sequence found in a respective portion of the synthetic peptide, is synthesized. In the synthesis of a fragment, if desired, incorporated may be an amino acid having its free amine (e.g., side chain amine) chemically protected by a chemical protecting agent. The fragments are then assembled (covalently coupled together in a manner and order) such that the synthetic peptide is produced (with the proper amino acid sequence). T20 (SEQ ID NO:3) was synthesized by fragment condensation approach, as previously described in more detail (see, e.g., U.S. Patent No. 6,015,881). Briefly, and as summarized in FIG. 4, fragments to be assembled into the synthetic peptide are first synthesized. A fragment comprising the first 16 amino acids of SEQ ID NO:3 was synthesized by standard solid phase synthesis (using a super acid sensitive resin), with acetylation ("Ac") of the N-terminus while having a hydroxyl group (-OH) at the C-terminus. A fragment comprising amino acids 17-26 of SEQ ID NO:3 was synthesized by standard solid phase synthesis with Fmoc at the N-terminus, and -OH at the C-terminus. A fragment comprising amino acids 27-35 of SEQ ID NO:3 was synthesized by standard solid phase synthesis with Fmoc at the N-terminus, and -OH at the C-terminus. As shown in FIG. 4, the fragment comprising amino acids 27-35 of SEQ ID NO:3 is chemically coupled to amino acid 36 in solution phase to result in a fragment comprising amino acids 27-36 with amidation of the C-terminus. The fragment of amino acids 17-26 of SEQ ID NO:3 was chemically coupled with the fragment of amino acids 27-36 of SEQ ID NO:3 (after removal of Fmoc from the N-terminal amino acid 27). The resulting amino acid sequence having amino acids 17-36 of SEQ ID NO:3 was chemically coupled with the fragment comprising amino acids 1-16 of SEQ ID NO:3 (after removal of Fmoc from the N-terminal amino acid 17) in forming a synthetic peptide comprising the amino acid sequence of SEQ ID NO:3. The synthetic peptide was deprotected/ decarboxylated (to remove tBU, trt, and Boc used in the synthesis of each fragment) with a deprotection step using a cocktail of trifluoroacetic acid/dithiothreitol/water (volume percent:90/5/5) at 30 degrees C, for 5 to 6 hours with stirring; and then purified using reverse-phase high performance liquid chromatography. Peptide identity was confirmed with electrospray mass spectrometry.

EXAMPLE 2

Illustrated is one embodiment of a method for site specific chemical modification of HIV gp41-derived peptide which may be used to produce (a) an isolated HIV gp41-derived peptide having a side chain amine group of one or more of its internal amino acids chemically protected; and (b) a substantially homogeneous conjugate comprised of

HIV gp41-derived peptide and polymer. More specifically, incorporated into a synthetic peptide (or a fragment thereof if a fragment assembly approach is used) during synthesis are: one or more amino acids having its side chain amine blocked by a chemical protecting agent from subsequent chemical reactivity with an amine-reactive functionality; and one or more amino acids having an amine (e.g., selected from the group consisting of N-terminal alpha amine, one or more epsilon amines, and a combination thereof) unprotected, and free for chemical reactivity with an amine-reactive functionality. An isolated synthetic peptide produced by this method for site-specific chemical modification can then be covalently coupled to polymer, in producing a substantially homogeneous conjugate, by chemically reacting the unprotected ("free") amine group(s) of the synthetic peptide to the amine-reactive functionality of a polymer. In this illustrative embodiment, T20 (SEQ ID NO:3) was selected as the exemplary synthetic peptide, and the lysine residue at amino acid position 18 ("K18") (an amino acid with a side chain amine) was chosen to be chemically protected by a site-specifically chemical modification, leaving lysine residue at amino acid position 28 ("K28") as the internal amino acid having a free amine. The free amine may subsequently be chemically reacted with an amine-reactive functionality of a polymer, in covalently coupling the polymer to the synthetic peptide via the lysine at amino acid position 28 of the amino acid sequence of the synthetic peptide. In referring to FIG.5, T20 (SEQ ID NO:3) was synthesized using the fragment condensation approach previously described in Example 1 herein.

Briefly, and as summarized in FIG. 5, a fragment comprising the first 16 amino acids of SEQ ID NO:3 was synthesized by standard solid phase synthesis with the N-terminal amine of amino acid residue 1 ("Y") being acetylated ("Ac"). A fragment comprising amino acids 17-26 of SEQ ID NO:3 was synthesized by standard solid phase synthesis using Fmoc-Lys-(ivDde) as amino acid residue 18, so that the chemical protecting agent ivDde ("X" in FIG. 5) blocks the epsilon amine group of K18 from reacting subsequently with an amine reactive functionality. A fragment comprising amino acids 27-35 of SEQ ID NO:3 was synthesized by standard solid phase synthesis, and chemically coupled to amino acid 36 in solution phase in forming a fragment comprising amino acids 27-36 of SEQ ID NO:3. The fragment comprising amino acids 17-26 of SEQ ID NO:3 (with the ivDde protected K18) was chemically coupled with the fragment of amino acids 27-36 of SEQ ID NO:3 (containing a lysine at position 28 ("K28") with a free epsilon amine). The resulting amino acid sequence comprising amino acids 17-36 of SEQ ID NO:3 was combined with the fragment comprising amino acids 1-16 of SEQ ID NO:3 in forming an isolated HIV gp41-derived peptide having the amino acid sequence of SEQ ID NO:3 and containing at least one amino acid having its side chain amine group chemically protected (blocked from subsequent chemical reactivity with an amine-reactive functionality) by a

chemical protecting agent. The synthetic peptide (SEQ ID NO:3) was deprotected to remove protecting groups trt, Boc, and tBu used in standard solid phase synthesis as described in more detail in Example 1 herein (while K18 remains chemically protected); decarboxylated; and then purified using reverse-phase high performance liquid chromatography. The isolated HIV gp41-derived peptide was then used for coupling a polymer site-specifically to the free epsilon amine of K28 of SEQ ID NO:3.

In producing a substantially homogeneous conjugate comprised of HIV gp41-derived peptide and polymer, mPEG succinimidyl propionate ("mPEG-SPA") was chosen as the exemplary polymer to conjugate to T20 (SEQ ID NO:3). T20 (SEQ ID NO:3) with ivDde on epsilon amine group of K18 (9.0 mg, 2.0 μ mol) was dissolved in dimethyl formamide (DMF)(0.3 ml). Diisopropylethylamine (DIEA) (10 μ l) was added to the reaction, and then added was mPEG-SPA (average molecular weight, 5000 daltons ("5K"); 20 mg, 4.0 μ mol) in DMF (1ml). The mixture was stirred at room temperature and the reaction is monitored by HPLC until the PEGylation was completed. To remove the chemical protecting agent ivDde from the epsilon amine group of K18, hydrazine (40 μ l) was added to the reaction to reach 3% (v/v) of hydrazine in reaction mixture. The stirring continued for another 30 minutes or until HPLC shows deprotection is completed. The reaction mixture was diluted by water (6.5ml) to make the final concentration of DMF at 20%, and then filtered through syringe filter (0.45 μ m, 2ml). HPLC purification was carried out on polystyrene/divinylbenzene column (PRLP-S, 300A, 10 μ m, 250*21.2mm) with acetonitrile-water-0.1% trifluoroacetic acid buffer as eluent. The collected fractions were checked by HPLC with both UV and ELS detectors. The pure fractions were pooled together and lyophilized for two days. The desired conjugate, a substantially homogeneous conjugate comprised of 5K-PEG-T20 at K28, was obtained as fluffy white solid (5.5mg) after lyophilization.

In another variation of this embodiment, the alpha amine of the N-terminal amino acid of SEQ ID NO:1 was not acetylated, but instead was protected with an Fmoc group. The process of synthetic peptide synthesis and conjugation to polymer was performed as provided in this Example 2. Thus, the resultant substantially homogeneous conjugate comprised 5K-PEG-T20 at K28, except that the T20 (SEQ ID NO:3) of the conjugate contained a free alpha amine at the N-terminal amino acid (Y).

EXAMPLE 3

Illustrated is another embodiment of a method for site specific chemical modification of HIV gp41-derived peptide which may be used to produce (a) an isolated HIV gp41-derived peptide having one or more internal amino acids with a side chain amine chemically protected; and (b) a substantially homogeneous conjugate comprised of HIV gp41-derived peptide and polymer. In this illustrative embodiment, T20 (SEQ ID

NO:3) was selected as the exemplary synthetic peptide, and the lysine residue at amino acid position 28 ("K28") was chosen to be chemically protected by a site-specific chemical modification, leaving lysine residue at amino acid position 18 ("K18") as the internal amino acid to be free for subsequent coupling to a polymer via the lysine side chain amine and an amine-reactive functionality of a polymer. In referring to FIG. 6, T20 (SEQ ID NO:3) was synthesized using the fragment condensation approach essentially as described in Example 1 herein. Briefly, and as summarized in FIG. 6, a fragment of SEQ ID NO:3 comprising the first 16 amino acids was synthesized by standard solid phase synthesis with the N-terminal amine of amino acid residue 1 (Tyr) being acetylated ("Ac"). A fragment of SEQ ID NO:3 comprising amino acids 17-26 was synthesized by standard solid phase synthesis. A fragment of SEQ ID NO:3 comprising amino acids 27-35 was synthesized by standard solid phase synthesis using Fmoc-Lys(ivDde) as amino acid residue 28 ("K28"), so that the chemical protecting agent ivDde ("X" in FIG. 6) blocks the epsilon amine group of K28 from subsequent chemical reactivity with an amine reactive functional group. The latter fragment was coupled to amino acid 36 in solution phase to form a fragment having amino acids 27-36 of SEQ ID NO:3. The fragment having amino acids 17-26 (containing K18 with a free epsilon amine) was combined with the fragment of amino acids 27-36 (with the ivDde protected K28). The resulting amino acid sequence having amino acids 17-36 was combined with the fragment comprising amino acids 1-16 in forming a synthetic peptide comprising the amino acid sequence of SEQ ID NO:3. The synthetic peptide (SEQ ID NO:3) was deprotected to remove protecting groups trt, Boc, and tBu used in standard solid phase synthesis as described in more detail in Example 1 herein (while K28 remains chemically protected); decarboxylated; and then purified using reverse-phase high performance liquid chromatography. The isolated HIV gp41-derived peptide was then used for coupling to a polymer site-specifically to the free epsilon amine of K18 of SEQ ID NO:3.

mPEG succinimidyl propionate ("mPEG-SPA") was chosen as the exemplary polymer to conjugate to T20 (SEQ ID NO:3). T20 (SEQ ID NO:3) with ivDde on epsilon amine group of K28 (19.7 mg, 4.4 μmol) was dissolved in DMF (0.5 ml). DIEA (20 μl) was added to the reaction, and then added was mPEG-SPA (average molecular weight, 5000 daltons ("5K"); 50 mg, 10 μmol) in DMF (1 ml). The mixture was stirred at room temperature and the reaction is monitored by HPLC until the PEGylation was completed. To remove the chemical protecting agent ivDde from the epsilon amine group of K28, hydrazine (45 μl) was added to the reaction to reach 3% (v/v) of hydrazine in reaction mixture. The stirring continued for another 30 minutes (or until HPLC shows deprotection is completed). The reaction mixture was diluted by water (6.5 ml) to make the final concentration of DMF at 20%, then filtered through syringe filter (0.45 μm, 2 ml). HPLC

purification was carried out on polystyrene/divinylbenzene column (PRLP-S, 300A, 10 μ m, 250*21.2mm) with acetonitrile-water-0.1% trifluoroacetic acid buffer as eluent. The collected fractions were checked by HPLC with both UV and ELS detectors. The pure fractions were pooled together and lyophilized for two days. The desired conjugate, a substantially homogeneous conjugate comprised of 5K-PEG-T20 at K18 was obtained as fluffy white solid (10.4mg) after lyophilization.

In another variation of this embodiment, the alpha amine of the N-terminal amino acid of SEQ ID NO:1 was not acetylated, but instead was protected with an Fmoc group. The process of synthetic peptide synthesis and conjugation to polymer was performed as provided in this Example 3. Thus, the resultant conjugate comprised a substantially homogeneous conjugate comprised of 5K-PEG-T20 at K18, except that the T20 (SEQ ID NO:3) of the conjugate contained a free alpha amine at the N-terminal amino acid (Y).

EXAMPLE 4

Illustrated in this example are: (a) a method determining the antiviral activity of substantially homogeneous conjugates produced according to the present invention; and (b) the need for a site-specific chemical modification according to the present invention to produce a substantially homogeneous conjugate comprised of polymer covalently coupled to HIV gp41-derived peptide. In using an *in vitro* assay for demonstrating antiviral potency, it is important to note that antiviral effect of synthetic peptide demonstrated in the *in vitro* assay has been correlated with the antiviral effect of the synthetic peptide *in vivo*. In determining antiviral activity (e.g., one measure being the ability to inhibit transmission of HIV to a target cell) of the synthetic peptide-polymer conjugates produced according to the present invention, used is an *in vitro* assay which has been shown, by data generated using synthetic peptides derived from either of the HR regions of HIV gp41, to be predictive of antiviral activity observed *in vivo*. More particularly, antiviral activity observed using an *in vitro* infectivity assay ("Magi-CCR5 infectivity assay"; see, e.g., U.S. Patent No. 6,258,782) has been shown to reasonably correlate to antiviral activity observed *in vivo* for the same HIV gp41-derived peptides. To further emphasize this point, T20 (SEQ ID NO:3) and T1249 (SEQ. ID NO:96) each have demonstrated potent antiviral activity against HIV in both the *in vitro* infectivity assay and human clinical trials.

The infectivity assays score for reduction of infectious virus titer employing the indicator cell lines MAGI or the CCR5 expressing derivative cMAGI. Both cell lines exploit the ability of HIV-1 tat to transactivate the expression of a β -galactosidase reporter gene driven by the HIV-LTR. The β -gal reporter has been modified to localize in the nucleus and can be detected with the X-gal substrate as intense nuclear staining within a

few days of infection. The number of stained nuclei can thus be interpreted as equal to the number of infectious virions in the challenge inoculum if there is only one round of infection prior to staining. Infected cells are enumerated using a CCD-imager and both primary and laboratory adapted isolates show a linear relationship between virus input and the number of infected cells visualized by the imager. In the MAGI and cMAGI assays, a 50% reduction in infectious titer ($V_n/V_o = 0.5$) is significant, and provides the primary cutoff value for assessing antiviral activity ("IC50" is defined as the dilution resulting in a 50% reduction in infectious virus titer). A secondary cutoff of $V_n/V_o = 0.1$, corresponding to a 90% reduction in infectious titer is also assessed ("IC90"). The substantially homogeneous conjugates tested for antiviral activity were diluted into various concentrations, and tested in duplicate or triplicate against an HIV inoculum adjusted to yield approximately 1500-2000 infected cells/well of a 48 well microtiter plate. The substantially homogeneous conjugate (in the respective dilution) was added to the cMAGI or MAGI cells, followed by the virus inocula; and 24 hours later, an inhibitor of infection and cell-cell fusion (e.g., T20) is added to prevent secondary rounds of HIV infection and cell-cell virus spread. The cells were cultured for 2 more days, and then fixed and stained with the X-gal substrate to detect HIV-infected cells. The number of infected cells for each control and substantially homogeneous conjugate dilution was determined with the CCD-imager, and then the IC50 and IC90 is calculated (typically expressed in $\mu\text{g/ml}$).

In this example, several substantially homogeneous conjugates, separately produced by the methods described herein, were analyzed for antiviral activity as shown in Table 1, and are identified as follows. "Conjugate A" is a synthetic peptide having the amino acid sequence of SEQ ID NO:3 having a 2K PEG site-specifically conjugated to the N-terminal amine (hence, the side chain amines of both K18 and K28 were chemically protected during synthesis). "Conjugate B" is a synthetic peptide having the amino acid sequence of SEQ ID NO:3 having a 2K PEG site-specifically conjugated to K18 (thus, the N-terminal amine and the side chain amine of K28 were chemically protected during synthesis). "Conjugate C" is a synthetic peptide having the amino acid sequence of SEQ ID NO:3 having a 2K PEG site-specifically conjugated to K28 (thus, the N-terminal amine and the side chain amine of K18 were chemically protected during synthesis). "Conjugate D" is a synthetic peptide having the amino acid sequence of SEQ ID NO:3 having a 5K PEG site-specifically conjugated to the N-terminal amine (hence, the side chain amines of both K18 and K28 were chemically protected during synthesis). "Conjugate E" is a synthetic peptide having the amino acid sequence of SEQ ID NO:3 having a 5K PEG site-specifically conjugated to K18 (thus, the N-terminal amine and the side chain amine of K28 were chemically protected during synthesis). "Conjugate F" is a

synthetic peptide having the amino acid sequence of SEQ ID NO:3 having a 5K PEG site-specifically conjugated to K28 (thus, the N-terminal amine and the side chain amine of K18 were chemically protected during synthesis).

5

Table 1

Molecule tested	Antiviral activity (IC ₅₀ µg/ml)
Synthetic peptide (SEQ ID NO:3)	< 0.01
Conjugate A	<0.02
Conjugate B	>0.05 <0.1
Conjugate C	>0.15
Conjugate D	>0.05 <0.1
Conjugate E	>0.3
Conjugate F	>0.4

From this comparison, it is clear that antiviral activity of the synthetic peptide is best preserved when a polymer of average size of 2K (2,000 daltons) is conjugated to the N-terminal amine versus at K18 (with at least 5 fold less activity) or K28 (with at least 10 fold less activity). Similarly, it is clear that antiviral activity of the synthetic peptide is best preserved when a 5K polymer is conjugated to the N-terminal amine versus at K18 (about 5 fold less activity) or K28 (about 7 fold less activity). Further, it can be concluded from this example, that the method of site specific chemical modification according to the present invention may be used to site-specifically couple a polymer to a selected amino acid of the synthetic peptide in producing a substantially homogeneous conjugate of peptide-polymer conjugate having a desired level of biological activity (e.g., in this example, desired antiviral activity is measured by having an IC₅₀ of less than 0.02 µg/ml) (e.g., "Conjugate A" of Table 1) while avoiding producing a synthetic peptide-polymer conjugate of multiple species that lacks the desired level of biological activity using standard PEGylation (e.g., a mixture of "Conjugate A" and "Conjugate B" and "Conjugate C" of Table 1).

EXAMPLE 5

In this example, illustrated are additional embodiments of the method for site-specific chemical modification of an HIV gp41-derived peptide, wherein incorporated into a synthetic peptide during synthesis are: one or more amino acids having its side chain amine blocked by a chemical protecting agent from subsequent chemical reactivity with an amine-reactive functionality; and one or more amino acids having an amine (e.g., selected from the group consisting of N-terminal alpha amine, one or more epsilon amines, and a combination thereof) unprotected, and free for chemical reactivity with an amine-reactive functionality. The resultant isolated HIV gp41-derived is then conjugated

to a polymer in producing a substantially homogeneous conjugate comprised of polymer and HIV gp-41 derived peptide.

Based on the teachings in Example 1 about a fragment condensation approach to synthesizing an HIV gp41-derived peptide, it is apparent to one skilled in the art that this approach of fragment assembly can be used, and has been used for some of the synthetic peptides having a sequence shown in SEQ. ID. NOs: 3-175, in the methods according to the present invention. Generally speaking, typically 3 fragments are synthesized (see, e.g., FIG. 3): an "N-terminal fragment" (usually comprised of between 10 and 20 of the amino acids of the amino terminus of the synthetic peptide), a "C-terminal fragment" (usually comprised of between 10 and 20 of the amino acids of the carboxy terminus of the synthetic peptide), and a "middle fragment" (usually comprised of between 10 and 20 of the amino acids found between the N-terminal fragment and the C-terminal fragment, in the synthetic peptide) which are then assembled to produce the complete synthetic peptide. However, depending on the length, the amino acid sequence, the number and location of amino acids with side chain amines in the amino acid sequence of a particular synthetic peptide, anywhere from 2 to 4 fragments have been synthesized, and then assembled to complete the synthesis of that particular synthetic peptide.

For example, T1249 (SEQ ID NO:96) was used in the method of site-specific chemical modification according to the present invention. In this example, the synthetic peptide was synthesized by the fragment condensation approach using 3 fragments: an N-terminal fragment comprising amino acids 1-12 and containing a lysine at amino acid position 7 ("K7"), and an acetylated N-terminal amino acid; a middle fragment comprising amino acids 13 to 26 and containing a lysine at amino acid position 21 ("K21"); and a C-terminal fragment comprising amino acids 27 to 36 and containing a lysine at amino acid position 28 ("K28") and a lysine at amino acid position 31 ("K31") (the amino acid position numbering corresponding to the respective positions in SEQ ID NO:96; i.e., in the assembled synthetic peptide). A number of isolated HIV gp41-derived peptides according to the present invention were separately produced: (a) a synthetic peptide having the amino acid sequence of SEQ ID NO:96 with a chemical protecting agent on K7, K21, K28, and K31 (leaving only the N-terminal amine free for subsequent conjugation to polymer); (b) a synthetic peptide having the amino acid sequence of SEQ ID NO:96 with a chemical protecting agent on the N-terminal amine, K21, K28, and K31 (leaving only K7 side chain amine free for subsequent conjugation to polymer); (c) a synthetic peptide having the amino acid sequence of SEQ ID NO:96 with a chemical protecting agent on the N-terminal amine, K7, K28, and K31 (leaving only K21 side chain amine free for subsequent conjugation to polymer); (d) a synthetic peptide having the amino acid

sequence of SEQ ID NO:96 with a chemical protecting agent on the N-terminal amine, K7, K21, and K31 (leaving only K28 side chain amine free for subsequent conjugation to polymer); and (e) a synthetic peptide having the amino acid sequence of SEQ ID NO:96 with a chemical protecting agent on the N-terminal amine, K7, K21, and K28 (leaving only K31 side chain amine free for subsequent conjugation to polymer).

Several substantially homogeneous conjugates were separately produced from these isolated HIV gp41- derived peptides having an amino acid sequence of SEQ ID NO:96 and the method for specific chemical modification described herein, using different sizes of PEG ranging from an average of 2K daltons ("2K") to an average of 20K daltons ("20K"). Table 2 shows antiviral activity of some of these substantially homogeneous conjugates, identified as follows. "Conjugate A" is a synthetic peptide having the amino acid sequence of SEQ ID NO:96 having a 2K PEG site-specifically conjugated to the N-terminal amine (hence, the side chain amines of K7, K21, K28, and K31 were chemically protected during synthesis). "Conjugate B" is a synthetic peptide having the amino acid sequence of SEQ ID NO:96 having a 2K PEG site-specifically conjugated to K7 (thus, the N-terminal amine, and the side chain amines of K21, K28, and K31 were chemically protected during synthesis). "Conjugate C" is a synthetic peptide having the amino acid sequence of SEQ ID NO:96 having a 2K PEG site-specifically conjugated to K21 (thus, the N-terminal amine, and the side chain amines of K7, K28, and K31 were chemically protected during synthesis). "Conjugate D" is a synthetic peptide having the amino acid sequence of SEQ ID NO:96 having a 2K PEG site-specifically conjugated to K28 (thus, the N-terminal amine, and the side chain amines of K7, K21, and K31 were chemically protected during synthesis). "Conjugate E" is a synthetic peptide having the amino acid sequence of SEQ ID NO:96 having a 2K PEG site-specifically conjugated to K31 (thus, the N-terminal amine, and the side chain amines of K7, K21, and K28 were chemically protected during synthesis). "Conjugate F" is a synthetic peptide having the amino acid sequence of SEQ ID NO:96 having a 5K PEG site-specifically conjugated to the N-terminal amine. "Conjugate G" is a synthetic peptide having the amino acid sequence of SEQ ID NO:96 having a 5K PEG site-specifically conjugated to K7. "Conjugate H" is a synthetic peptide having the amino acid sequence of SEQ ID NO:96 having a 5K PEG site-specifically conjugated to K21. "Conjugate I" is a synthetic peptide having the amino acid sequence of SEQ ID NO:96 having a 5K PEG site-specifically conjugated to K28. "Conjugate J" is a synthetic peptide having the amino acid sequence of SEQ ID NO:96 having a 5K PEG site-specifically conjugated to K31.

Table 2

Molecule tested	Antiviral activity (IC ₅₀ µg/ml)
Synthetic peptide (SEQ ID NO:96)	< 0.01
Conjugate A	<0.01
Conjugate B	<0.01
Conjugate C	>0.01
Conjugate D	<0.01
Conjugate E	>0.01
Conjugate F	>0.03
Conjugate G	>0.04
Conjugate H	>0.04
Conjugate I	>0.03
Conjugate J	>0.04

From this example, and in viewing the results using a PEG of average size of 2K (2000 daltons), it can be concluded that the method of site-specific chemical modification according to the present invention may be used to site-specifically couple a polymer to a selected amino acid of the synthetic peptide in producing a substantially homogeneous conjugate having a desired level of biological activity (e.g., in this example, the desired antiviral activity is measured by having an IC₅₀ of less than 0.01 µg/ml) (e.g., "Conjugate A", "Conjugate B", and "Conjugate D" of Table 2) while avoiding producing a synthetic peptide-polymer conjugate of multiple species that lacks such desired level of biological activity using standard PEGylation (e.g., a mixture of the Conjugates A-E of Table 2).

EXAMPLE 6

In another example, a synthetic peptide having an amino acid sequence of SEQ ID NO:174 was used in the method of site-specific chemical modification according to the present invention. In this example, the synthetic peptide containing a lysine at amino acid position 30 ("K30") and a lysine at amino acid position 39 ("K39"; the C-terminal amino acid; the amino acid position numbering corresponding to the position in SEQ ID NO:174) was synthesized by linear synthesis with a chemical protecting agent on the N-terminal amine of the N-terminal amino acid, and on the side chain amine of K30 (leaving only the K39 side chain amine free for subsequent conjugation to polymer). Substantially homogeneous conjugates were separately produced from this isolated HIV gp41- derived peptide using a polymer having an average size of 2K, a polymer having an average size of 5K, and a polymer having an average size of 20K. For example, the synthetic peptide alone (not conjugated to a polymer) has a desired level of biological activity (e.g., antiviral activity as measured by having an IC₅₀ of less than or equal to 0.02 µg/ml), whereas a substantially homogeneous conjugate synthetic having a polymer of average size of 2K had biological activity about equal to 0.02 µg/ml. Substantially homogeneous conjugates

having a polymer of average size of either 5K or 20K had biological activity much greater than 0.1 µg/ml (i.e., outside the range of desired biological activity for this example).

EXAMPLE 7

5 The present invention provides for substantially homogeneous conjugates comprised of an HIV gp41-derived peptide to which is coupled site-specifically a polymer ("synthetic peptide-polymer conjugates"). Antiviral activity of such synthetic peptide-polymer conjugates can be utilized in a method for inhibiting transmission of HIV to a target cell, comprising adding to the virus and cell an amount of synthetic peptide-polymer
10 conjugate according to the present invention effective to inhibit infection of the cell by HIV, and more preferably, to inhibit HIV-mediated fusion between the virus and the target cell. This method may be used to treat HIV-infected individuals (therapeutically) or to treat individuals newly exposed to or at high risk of exposure (e.g., through drug usage or high risk sexual behavior) to HIV (prophylactically). Thus, for example, in the case of an
15 HIV-1 infected individual, an effective amount of synthetic peptide-polymer conjugate would be a dose sufficient (by itself and/or in conjunction with a regimen of doses) to reduce HIV viral load in the individual being treated. As known to those skilled in the art, there are several standard methods for measuring HIV viral load which include, but are not limited to, by quantitative cultures of peripheral blood mononuclear cells and by
20 plasma HIV RNA measurements. The synthetic peptide-polymer conjugates of the invention can be administered in a single administration, intermittently, periodically, or continuously, as can be determined by a medical practitioner, such as by monitoring viral load. Depending on the formulation containing synthetic peptide-polymer conjugate, and such factors as the compositions of the polymer and synthetic peptide used in forming the
25 synthetic peptide-polymer conjugate and whether or not further comprising a pharmaceutically acceptable carrier and the nature of the pharmaceutically acceptable carrier, the synthetic peptide-polymer conjugate according to the present invention may be administered with a periodicity ranging from days to weeks or possibly longer. Further, a synthetic peptide-polymer conjugate according to the present invention may be
30 used, in antiviral therapy, when used in combination or in a therapeutic regimen (e.g., when used simultaneously, or in a cycling on with one drug and cycling off with another) with other antiviral drugs used for treatment of HIV (e.g., including, but not limited to, other HIV entry inhibitors (e.g., CCR5 inhibitors, retrocyclin, and the like), HIV integrase inhibitors, reverse transcriptase inhibitors (e.g., nucleoside or nonnucleoside), protease
35 inhibitors, viral-specific transcription inhibitors, viral processing inhibitors, HIV maturation inhibitors, inhibitors of uridine phosphorylating enzyme, HIV vaccines, and the like, as well known in the art.

For example, in one preferred embodiment, one or more antiviral agents may be combined in therapy with synthetic peptide-polymer conjugate according to the present invention, thus increasing the efficacy of the therapy, and lessening the ability of the virus to become resistant to the antiviral drugs. Such combinations may be prepared from effective amounts of antiviral agents (useful in treating of HIV infection) currently approved or approved in the future, which include, but are not limited to, abacavir, AZT, delaviridine, ddC, ddI, efavirenz, FTC, (+) and (-) FTC, Reverset, GS 840, HBY097, 3TC, nevirapine, d4T, FLT, emtricitabine, amprenavir, CGP-73547, CGP-61755, DMP-450, indinavir, nelfinavir, PNU-140690, ritonavir, saquinavir, telinavir, tenofovir, adefovir, atazanavir, lopinavir, VX 478, PRO-542, and betulin and dihydrobetulin derivatives (e.g., PA-457). Effective dosages of these illustrative antiviral agents, which may be used in combinations with synthetic peptide-polymer conjugate according to the present invention, are known in the art. Such combinations may include a number of antiviral agents that can be administered by one or more routes, sequentially or simultaneously, depending on the route of administration and desired pharmacological effect, as is apparent to one skilled in the art.

Effective dosages of a synthetic peptide-polymer conjugate of the invention to be administered may be determined through procedures well known to those in the art; e.g., by determining potency, biological half-life, bioavailability, and toxicity. In a preferred embodiment, an effective synthetic peptide-polymer conjugate dosage range is determined by one skilled in the art using data from routine *in vitro* and *in vivo* studies well known to those skilled in the art. For example, *in vitro* infectivity assays of antiviral activity, such as described herein, enables one skilled in the art to determine the mean inhibitory concentration (IC) of the synthetic peptide-polymer conjugate necessary to block some amount of viral infectivity (e.g., 50% inhibition, IC₅₀; or 90% inhibition, IC₉₀). Appropriate doses can then be selected by one skilled in the art using pharmacokinetic data from one or more standard animal models, so that a minimum plasma concentration (C_{min}) of the synthetic peptide-polymer conjugate is obtained which is equal to or exceeds a predetermined IC value. While dosage ranges typically depend on the route of administration chosen and the formulation of the dosage, an exemplary dosage range of the synthetic peptide-polymer conjugate according to the present invention may range from no less than 0.1 µg/kg body weight and no more than 10 mg/kg body weight; preferably a dosage range of from about 0.1-100 µg/kg body weight; and more preferably, a dosage of between from about 10 mg to about 250 mg of synthetic peptide-polymer conjugate.

A synthetic peptide-polymer conjugate of the present invention may be administered to an individual by any means that enables the active agent to reach the

target cells (cells that can be infected by HIV). Thus, the synthetic peptide-polymer conjugates of this invention may be administered by any suitable technique, including oral, parenteral (e.g., intramuscular, intraperitoneal, intravenous, or subcutaneous injection or infusion, intradermal, or implant), nasal, pulmonary, vaginal, rectal, sublingual, or topical routes of administration, and can be formulated in dosage forms appropriate for each route of administration. The specific route of administration will depend, e.g., on the medical history of the individual, including any perceived or anticipated side effects from such administration, and the formulation of conjugate being administered (e.g., the nature of the polymer and synthetic peptide of which the synthetic peptide-polymer conjugate comprises). Most preferably, the administration is by injection (using, e.g., intravenous or subcutaneous means), but could also be by continuous infusion (using, e.g., slow-release devices or minipumps such as osmotic pumps, and the like). A synthetic peptide-polymer conjugate according to the present invention may further comprise a pharmaceutically acceptable carrier; and may further depend on the formulation desired, site of delivery, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

The foregoing description of the specific embodiments of the present invention have been described in detail for purposes of illustration. In view of the descriptions and illustrations, others skilled in the art can, by applying current knowledge, readily modify and/or adapt the present invention for various applications without departing from the basic concept; and thus, such modifications and/or adaptations are intended to be within the meaning and scope of the appended claims.

What is claimed is: